A novel thymol-doped enamel bonding system: Physico-mechanical properties, bonding strength, and biological activity

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Abstract: PURPOSE Over the past decades, the preparation of antibacterial restorative dental adhesives has obtained increasing attention in order to prevent secondary caries. In the present study, a novel essential oil-based antibacterial resin adhesive was prepared and evaluated for dental applications. In this regards, thymol, which is a major phenolic component of thyme essential oil, was incorporated into methacrylate resin matrix and its effect on the physico-mechanical and biological properties of the experimental bonding agent was investigated. MATERIALS AND METHODS Mechanical properties were evaluated via measuring flexural strength, flexural modulus and fracture toughness. Degree of conversion (DC%) of monomers was measured using FTIR spectroscopy. Viscoelastic properties of the samples were also determined by dynamic mechanical thermal analysis (DMTA). The bactericidal activity of composite specimens against Streptococcus mutans (ATCC 35668) was determined based on ASTM E 2180-07.MTT assay was performed to investigate the cytocompatibility of samples. Furthermore, the bonding strength of the adhesives was evaluated through microshear bond test on the caries-free extracted human premolar teeth and the mode of failure was investigated by scanning electron microscopy. RESULTS Thymol-doped resin adhesive exhibited comparable degree of conversion to the control resin adhesive. The plasticizing behavior of thymol slightly decreased the flexural modulus and glass transition temperature of the thymol containing specimens, even though; it caused significant increases in fracture toughness of adhesive. The results represented appropriate antibacterial activity as well as suitable cytocompatibility. Furthermore, the thymol-doped resin adhesive showed comparable adhesive strength to the control. CONCLUSION The thymol is extremely compatible with the methacrylate resin restorative system and completely fulfills all requirements of a good bactericidal component in construction of an ideal enamel bonding system.

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A novel thymol-doped enamel bonding system: Biological activity, adhesive strength and physico-mechanical properties

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Short title: *Thymol doped enamel bonding*

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ABSTRACT

Purpose*:* Over the past decades, the preparation of antibacterial restorative dental adhesives has obtained increasing attention in order to prevent secondary caries. In the present study a novel essential oil-based antibacterial resin adhesive was prepared and evaluated for dental applications. In this regards, thymol, which is a major phenolic component of thyme essential oil, was incorporated in methacrylate resin matrix and samples were cured through photopolymerization reaction.

Materials and Methods: Mechanical properties were evaluated via measuring flexural strength, flexural modulus and fracture toughness. Degree of conversion (DC%) of monomers was measured using FTIR spectroscopy. Viscoelastic properties of the samples were also determined by dynamic mechanical thermal analysis (DMTA). The bactericidal activity of composite specimens against Streptococcus mutans (ATCC 35668) was determined based on ASTM E 2180 – 07. MTT assay was performed to investigate the cytocompatibility of samples. Furthermore, adhesive strength of bonding systems was evaluated through microshear bond test on the caries-free extracted human premolar teeth and the mode of failure was investigated by scanning electron microscopy.

Results: Thymol-doped resin adhesive exhibited comparable degree of conversion to the control resin adhesive. The plasticizing behavior of thymol slightly decreased the flexural modulus and glass transition temperature of samples, even though; it caused significant increases in fracture toughness of adhesive. The results represented appropriate antibacterial activity as well as suitable cytocompatibility. Furthermore, the thymol-doped resin adhesive showed comparable adhesive strength to the control.

Conclusion: The thymol is extremely compatible with the methacrylate resin restorative system and completely fulfills all requirements of a good bactericidal component in construction of a ideal enamel bonding system.

Keywords*:* Antibacterial activity; Dental resin adhesive; Microshear bond test; Thymol

1. Introduction

Dental restorative materials have undergone great progress in the past decades. In this regard, resin-based dental composites and adhesives are currently utilized to overcome the short comings which esthetically associated with dental amalgam (Beigin et al., 2013). Dental adhesive are used to establish a strong and durable bond between composite fillings and tooth structure (Marshall et al., 2010). The formulation of dental adhesives and polymeric composites generally are based on acrylic/methacrylate resin monomers which polymerized through light curing (Breschi et al., 2008). Methacrylate monomers suffer from some drawbacks such as shrinkage stress and shrinkage strain which may result in bond failure between the composite and adhesive or formation a marginal gap in the adhesive-tooth interface (Van Ende A et al., 2010). These phenomena may provide a good environment for bacterial activity and plaque accumulation which result in secondary caries and restoration failure (Burunjeny et al., 2015). Over the past decades, the preparation of antibacterial dental materials has been taken into consideration in order to prevent secondary caries (Burunjeny et al., 2017). In this regard, many different bactericidal components have been incorporated in dental materials. In addition to exhibit suitable bactericidal activity, there are some criteria that should be fulfilled by bactericidal agents. Proper cytocompatibility, low water uptake, good miscibility, color matching and not scarifying the mechanical properties are some of the main requirements of the ideal bactericidal componentes (Cocco et al., 2015). Planet naturally derived antibacterial components have recently obtained growing research in different fields to explore the possibility of substitution of synthetic bactericidal products which lots of concerns regarding public health have currently raised about (Bassolé et a., 2012). In this regard, essential oils and their constituents have obtained a great deal of attention as bactericidal components and are widely utilized due to their broad spectrum antibacterial activity, safety in terms of human health and availability at a low cost. Furthermore, their adopted complex chemical composition can decrease the risk of increasing bacteria resistance (Seow et al., 2014). Thymol, which is a major phenolic component of thyme essential oil, has promising characteristic for treatment of root caries and oral therapy (Marchese et al., 2016). However, to our knowledge, the use of thymol has not been taken into account in dental restorative materials. Hence, in the present study a novel thymol-based antibacterial resin adhesive was prepared and evaluated for dental

applications. In this regard, thymol was incorporated in methacrylate resin matrix and samples were cured through photopolymerization reaction. The main hypothesis was that the structural characteristics of thymol not only lead to acceptable antibacterial activity, color matching and good miscibility with methacrylate resin, but also its slight water solubility gives rise to sustained release and consequently long lasting antibacterial activity. The validity of our hypothesis was examined by investigation of biological activity and physico-mechanical properties of thymol-incorporated dental adhesive.

2. Material and methods

Materials

Camphorquinone (CQ), N,N-dimethylaminoethyl methacrylate (DMAEMA), 2, 2-Bis-(2-hydroxy-3-methacryloxypropoxy) phenyl propane (Bis-GMA) and triethyleneglycoldimethacrylate (TEGDMA) were kindly provided by Evonik (Germany) and Thymol was purchased from Merck (Germany). The 37.5% phosphoric acid gel (Kerr Gel Etchant) was obtained from SDS Kerr (USA). DENU-Composite Resin, a commercially available light curing universal composite resin with nano hybrid filler, was obtained from HDI Inc (Korea). Porcelain Bonding Resin, commercially available light curing unfilled enamel bonding, was obtained from Bisco Inc (USA).

Characterization

Degree of conversion

The degree of conversion of methacrylate functions was followed using FTIR spectroscopy (Equinox 55Bruker instrument (Bruker, Germany). The thin resin specimens were placed between two polyethylene films to prevent oxygen inhibition during photopolymerization and photopolymerized using the light curing unit. DC was evaluated by comparing the absorbance spectrum of uncured methacrylate double bond (peak at 1638 cm^{-1}) before and after 100 s curing of the specimen (Beigi et al., 2013). The spectrum of aromatic carbon–carbon double bond (peak at 1608 cm^{-1}) was used as internal reference. The degree of conversion was then calculated as follows:

$$DC\% = (1 - \frac{(1636/1608 cm^{-1})\, peak\ area\ after\ curing}{(1636/1608 cm^{-1})\, peak\ area\ before\ curing}) \times 100\%$$

Flexural properties

Flexural strength of the samples was measured according to the 3-point bending method carried out with a universal test machine (STM-20, Santam, Iran) at a cross-head speed of 1 mm/min. The bar specimens were prepared in dimensions of 2 mm × 2 mm × 25 mm according to ISO 4049 (Standard I, 2000). The specimens were irradiated for 100s on both sides with a light-curing unit (Optilux 501, Kerr, USA) at an intensity of 550 mW.cm^{-2}. The specimens were stored in distilled water at 37 °C for 24h prior to testing. The flexural strength (FS) in MPa was then calculated as:

$$FS = {}^{3PL}/_{2bd^2}$$

Where P stands for load at fracture (N), L is the span length (20 mm), and b and d are, the width and thickness of the specimens in mm, respectively. The elastic modulus was also determined from the slope of the initial linear part of stress–strain curve.

Fracture toughness

Fracture toughness of a material is determined from the stress intensity factor (*K*) during crack propagation. To determine the fracture toughness (FT), single-edge notch beam specimens were fabricated according to ASTM Standard E399-90 in a 5mm×2mm×25mm split steel mold with a razor blade providing a 2.5 mm notch in the middle of the specimens (Designation, 1997). The bending fracture test was performed at a cross-head speed of 0.1 mm.min^{-1} using the universal test machine and the fracture toughness (critical stress intensity factor, 0.1 K_{IC}) was calculated according the following equation:

$$K_{IC} = \frac{3PL}{2BW^{3/2}} \left\{ 1.93\left(\frac{a}{W}\right)^{1/2} - 3.07\left(\frac{a}{W}\right)^{3/2} + 14.53\left(\frac{a}{W}\right)^{5/2} - 25.11\left(\frac{a}{W}\right)^{7/2} + 25.8\left(\frac{a}{W}\right)^{9/2} \right\}$$

Where *P* is load at fracture (N), *L*, *W*, *B*, and *a*, are length, width, thickness, and notch length (in mm), respectively. The span length and load cell capacity were 20 mm and 60 N, respectively. The subscript *Ic* denotes mode I crack opening under a normal tensile stress perpendicular to the crack (Soderholm, 2010).

Viscoelastic properties:

Viscoelastic properties of the samples were investigated by dynamic mechanical analysis (DMA) using a UK Polymer Lab model MK-II analyzer in bending mode over the temperature range from −100 to 200 ◦C at a heating rate of 5 ◦C min^{-1} and frequency of 1Hz. The loss tangent was recorded as a function of temperature and glass transition temperature (T_g) was taken of the maximum of loss tangent curve. $T_{g1/2width}$ was also measured from the half-peak width of loss tangent curves.

Microshear bond test

49 caries-free extracted human premolar teeth were collected in conformity with the rules and guidelines of the Research Ethics Committee of Science and Research Branch, Islamic Azad University, Tehran, Iran with the code **IR.IAU.SRB.1396.70**. All teeth were washed under running water to remove tissue remnants and debris and stored in formaldehyde 10% for up to 3 months. A week before the test the teeth were cut and grinded using a trimmer (Dentaurim, Germany) and silicon carbide paper (600 grits) respectively. The teeth slices (approximately 3.0mm thick) with a polished flat bonding site on an enamel surface were prepared and used in determination of microshear bond strength of dental adhesives. In this regard, the resin adhesives were applied on the tooth slice surface and then a commercially available composite (DENU-Composite Resin, Korea) was molded into cylinder shape (internal diameter: 1.0mm, height: 1.0mm) and was placed on the adhesive thin layer and light cured for 40s. A thin orthodontic wire was looped around the composite cylinder as close as possible to the tooth-cylinder interface and a shear force was applied to cylinder using a universal testing machine until failure occurred. The bond strength, which is known as nominal strength, is obtained by dividing the load at failure by the cross sectional area along adhesive bonded interface.

Scanning electron microscopy:

The fractured surface along bonded interface was observed using SEM (TESCAN, VEGAII, XMU, Czech Republic) in order to investigate the morphology and to study the mode of failure after microshear bond test.

Antibacterial activity

The biocidal activity of adhesive specimens containing 5% wt thymol was determined based on ASTM E 2180 – 07 and against Streptococcus mutans (ATCC 35668). Briefly, the disc shaped adhesives (1 cm

diameter) after photocuring were aged in 37◦C sterilized phosphate buffered saline for 1 day and 4 months. In the following, 100 microliters of the molten agar slurry, which pre-inoculated with Streptococcus mutans suspension, was then placed on the samples (approximately 10_6 CFU/ml of bacteria) in 24-well micro titer plate and the plate was incubated for 24 h in a humid anaerobic atmosphere at 37◦C. During the incubation period, the suspension liquid, evaporated and a thin layer of pre-inoculated agar was maintained in direct contact with the samples. After the specified contact time, neutralizing broth (1ml) was then added to each well and agar slurry inoculums were recovered from the test specimens through gentle pipetting for 2 min. A serial tenfold dilution was made with BHI broth and each suspension was placed on blood agar plates and incubated for 48 h. Microbial colonies of each plate was then counted and recorded. The percentage of microorganism reduction was calculated according to the following equation:

$$Geometric\ mean = \frac{Log_{10}X_1 + Log_{10}X_2 + Log_{10}X_3}{3}$$

$$Reduction\% = \frac{(a-b) \times 100}{a}$$

,where (x) assigns number of bacteria recovered from the incubation period control or incubation period treated samples, (a) designates the antilog of the geometric mean of organisms recovered from the incubation period control samples and (b) designates the antilog of the geometric mean of organisms recovered from the incubation period treated samples.

Cytocompatibility

Cytocompatibility of adhesive samples was evaluated against L929 fibroblast cell by either microscopic investigation of cells morphology after direct contact with samples or tetrazolium dye-based colorimetric assay (MTT assay). The photocured adhesives were sterilized by incubation at 120 ºC for 15 min. The former test method was evaluated according procedure reported in our previously published article (Burunjeny et al., 2015). The viability of the fibroblast cells contacted with samples was performed by MTT

assay according to ISO 10993-5 (10993-5, 2009). The percentage of relative cell viability was calculated according to following equation:

$$Cell\ viability\% = \frac{OD_{Sample} - OD_{Positive\ control}}{OD_{Negative\ control}}$$

, Where OD is the mean value of the measured optical density at 540 nm.

Thymol-release test:

The release of thymol from the photocured specimens was performed during 4 months incubation at 37◦C in contact with sterilized phosphate buffered saline. To this end, six photocured cylindrical specimens were prepared in a diameter of 20 mm and a thickness of 2 mm (Each disk weights about 200mg and contains 5wt. % of thymol). The samples were suspended in 10 ml of PBS and incubated at 37 °C. At each time point, 3 ml of PBS solution was withdrawn and the presence of thymol was monitored at 274 nm spectrum using an ultraviolet spectrophotometer. After each sampling interval, the collected volume of PBS was compensated by fresh PBS solution and so at the various time the previously thymol absorption was taken into consideration to calculate total thymol concentration.

Statistical analysis

The results (five repeats for the mechanical properties and degree of conversion and three repeats for bactericidal assay) were analyzed and compared using one-way ANOVA and the Tukey test at the significance level of 0.05.

3. Results

Different formulations of dental adhesive are shown in Table 1. All formulations contain 1wt.% CQ and 0.5wt%BD as photoinitiator system and BisGMA/TEGDMA content was kept constant at 70/30 wt.%.

Degree of conversion of methacrylate moieties (DC %) are tabulated in Table 1. This measurement revealed the same DC% values about 60 for adhesives with no significant differences ($P > 0.05$).

The values of flexural properties of light-cured dental adhesives are depicted in Figure 1 a. Thymol-doped sample showed lower flexural strength and flexural modulus in comparison to neat methacrylate specimen. However, there is not a significant difference among the neat adhesive and thymol-doped adhesive neither in flexural modulus nor flexural strength ($P > 0.05$).

The values of fracture toughness for light-cured dental adhesives are shown in Figure 1 b. Incorporation of thymol gave rise to no significant increase in fracture toughness ($P > 0.05$).

Figure 2a shows loss tangent versus temperature for specimens. The parameters including T_g and $T_{g1/2width}$ of samples were extracted from loss tangent curves and collected in Table 1. Thymol incorporated sample showed lower T_g and $T_{g1/2width}$ in comparison to neat methacrylate analogous.

Figure 3a shows the microshear bond strength of the adhesive specimens. It reveals no significant difference in microshear bond strength between thymol incorporated sample and neat methacrylate specimen ($P > 0.05$). Furthermore, there is no significant difference between thymol-doped adhesive and commercially available bonding systems. The typical SEM micrographs of the enamel surface before and after microshear bond fracture are depicted in Figure 3 b and c respectively. Figure 3c illustrates the failure mode of the deboned surface during microshear bond test. This figure shows adhesive failures mode for the samples containing thymol.

The bactericidal activity of light-cured dental adhesive containing 5% wt thymol after 1 day and 4 months of aging in phosphate buffered saline was evaluated and quantified by colony count method. Figure 4 show the respective images of blood agar plate culture of harvested S.mutance bacteria after 24h in direct contact to aged samples and the recorded results of Log reduction in CFU are collected in Table 1. As it represents the antibacterial activity of sample slightly reduces after 4 months of aging, even though, the bactericidal activity is in the acceptable range for using in dental applications.

The MTT assay was performed directly on light-cured adhesives to evaluate their cytocompatibility against L929 mouse fibroblast cells. The optical microscopy images of L-929 cells in direct contact with the photocured adhesive samples after 48 h incubation were depicted in Figure 5. According to these images, all fibroblast cells survived and kept their spindle shape morphology and grew to confluence in the vicinity of photocured adhesives with no cell lysis. Furthermore, the viability of L929 mouse fibroblast cells which

cultured with photocured specimens was collected in Figure 5 d. It can be concluded from these results, that the viability of cells cultured on adhesives surface is in the range of 90-100% and there are no statistically significant differences ($P > 0.05$) between viability values of neat and thymol incorporated adhesives with the negative control.

The thymol release profile is shown in figure 6. As it appears, a sustained release was observed during 4 months which reached a thermodynamic equilibrium at thymol concentration about 10 mg/l.

4. Discussion

Over the past decades the concept of “minimally invasive dentistry” has attracted a great deal of attention. The philosophy of minimally invasive dental care can be defined as maximal preservation of healthy dental structures by preventing oral disease from occurring or by intercepting and eradicating those diseases and prevent their recurrence with minimal tissue loss (Murdoch-kinch and McLean, 2003). One of the most important MID guidelines established based on the fact saying ‘prevention is better than cure’. In this regard, “Materials science” is one the most crucial factors which plays the main role in development of MID approach (Frencken et al., 2012). For example antibacterial dental restorative materials have been used to prevent the secondary caries after primary dental treatment. In this study a new antibacterial dental adhesive based on essential oil has been developed.

The progress of polymerization reaction and network formation was evaluated by measurement of methacrylate group conversion (DC %). In this regard, the disappearance of the methacrylate doubles bonds was followed by monitoring the FT IR absorbance spectrum at 1638 cm^{-1}, either in the absence or in the presence of thymol. As it appears in Table 1, the thymol-doped adhesive exhibited comparable degree of conversion to the control resin adhesive. So, although most of phenolic compounds are known to act as an inhibitor of radical polymerization (Fujisawa and Kadoma, 1992; Dossot et al., 2000), the presence of thymol does not affect significantly the progress of polymerization. Thus, it can be concluded that thymol can be completely compatible with the methacrylate resin restorative system. For more illustration of mixing compatibility of thymol, the direct visual inspection of the photocured specimens was evaluated. As

it can be seen in Figure 2b incorporation of thymol does not change the transparency of photocured sample. So this observation not only shows the good miscibility of thymol with methacrylate monomers, but also it can be concluded that no phase separation occurs through photopolymerization.

As it shown in Figure 1a incorporation of thymol give rise to statistically nonsignificant decrease of flexural properties ($p > 0.05$). On the other hand as it already mentioned introducing of thymol does not affect the conversion of methacrylate functional groups. According to these observations it can be concluded that thymol acts such as a plasticizer which can reduce the intermolecular attraction of polymeric chains and thereby slightly decreasing flexural properties (Degli Esposti et al., 2013).

Fracture toughness of materials is defined as the plastic energy dissipation ability and thereby mechanical resistance to fracture and crack propagation (Soderholm, 2010). Considering that the dental restorative materials are subjected under mastication forces, their fracture toughness plays the main role in their longevity (Watanabe et al., 2008). As shown in Figure 1b the fracture toughness of dental adhesive enhances with incorporation of thymol. This observation is due to plasticizing effect of thymol that facilitates the polymer chains sliding over one another and consequently increases the plastic deformation and energy dissipating during crack propagation and thereby increasing fracture toughness (Balkenhol et., 2008).

For more elucidation of miscibility and plasticizing effect of thymol, the glass transition temperature and structure homogeneity of dental adhesives were studied through dynamic mechanical thermal analysis. As shown in figure 2a with introducing thymol into resin adhesive, the maximum of the loss tangent shifted to lower temperature. This observation can be attributed to plasticizing behavior of thymol which eases the polymeric chain movement and consequently slightly decreases the glass transition temperature. The visual inspection of thymol-doped photocured adhesive showed the transparent structure which implies good miscibility of thymol with methacrylate monomers and thereby formation a homogenius photocrosslinked network with no phase separation. The dynamic mechanical thermal analysis also can be used as a complementary method for studying the homogeneity of specimens (Menard, 2008). In this regard, the investigation of thermograms reveal that incorporation of thymol decreased the peak width of

loss tangent curve which is a result of higher degree of structural homogeneity of the thymol-doped photocured adhesive. Actually, the width of the loss tangent curve is depended on the nature of the thymol-polymer interaction. As a matter of fact, a narrower single loss tangent curve implies the more homogeneous structure with no separation domains (Beigi et al., 2013; Menar, 2008).

Dental adhesives play the main role in creation a strong and durable bond between tooth cavity and composite restoration. In this regard, evaluation of the bond strength of adhesive systems has been given prime importance in formulation and development of dental adhesives (Armstrong et al., 2010). Microshear bond test is one of the straight forward mechanical test methods used to evaluate the bond strength of bonding system (Placido et al., 2007; Kanat et al., 2014). The microshear test results presented in Figure 3a demonstrate that incorporation of thymol into adhesive does not change the microshear bond strength significantly ($p > 0.05$). Furthermore, the value of microshear bond strength for the commercially available bonding resin is comparable to thymol doped adhesive specimen. Applying the pure shear force at the point of loading is a crucial factor in evaluating the usefulness of microshear bond test. On the other hand, the mixed mode loading generated along the bonded interface, including shear and tensile forces, is one of the most challenges to the validity of microshear bond test (El Zohairy et al., 2010). Furthermore, the uniformity of stress distribution across the bonded interface is very important to prevent the stress concentration which may result in premature cohesive failure (Marshall et al., 2010). In order to fulfill the mentioned requirements, the shear force is applied as close as possible to the bonded interface by using a thin orthodontic wire .However; it should be made clear that microshear bond test is not without their limitation. Studying the mode of failure as another important aspect of micro shear bond test may indicate the validity of this test (El Zohairy et al., 2010). In this regard electron microscopical study of the fractured surface (Figure 3c) revealed mostly adhesive failure from adhesive-enamel interface. According to this observation it can be concluded that the stress state generated along the bonded interface is mainly arisen from the shear force as external stimuli.

According to the World Health Organization, dental restoration replacement has increased despite the improvement in oral health. Secondary caries, which is known as a silent epidemic, has been the main reason of secondary restoration-replacement (Petersen and Ogawa, 2016; Petersen, 2008). Since this

problem arisen from oral bacterial activity in marginal gap or dental plaque accumulation, so introducing antibacterial components can be used to prevent secondary caries. Thymol is a planet-based monoterpene phenol with antibacterial activity against both Gram-positive and Gram-negative bacteria (Median et al., 2019). On the other hand, since Streptococcus mutans is known as a primary pathogen of dental caries, the antibacterial activity of thymol-doped dental adhesive was performed against s.mutans through direct contact test. The results of this test for 1-day aged sample exhibited strong bactericidal activity. In fact this sample could kill 92% of CFU of bacteria. According to Figure 4 and Table 1 the antibacterial activity of 4-months aged sample slightly decreased and killed 84% of CFU of bacteria. In order to assess the long-term antibacterial effect, the time-dependent release of thymol from the photo cured adhesive was monitored. The release profile (Figure 6) exhibited a sustained release for thymol which reached a thermodynamic equilibrium at thymol concentration about 10 mg/l. This finding confirms the long-lasting antibacterial effectiveness of thymol which is attributed to the hydrophobic nature and thereby gradually solubility of thymol in aqueous oral cavity environment. This characteristic leads to slowly release thymol at enough oral bioavailability.

Actually, the action mode of antibacterial activity of thymol is strongly affected by the physico-chemical characteristic of the phenolic aromatic ring (Marchese et al., 2016). Although hydrophobic characteristic of thymol decreases the water solubility, its aqueous concentration is enough to migrate across the aqueous extracellular environment and to interact with bacteria membrane. It is speculated that the strong lipophilic nature of thymol plays the main role in accumulation in the bacteria membrane and consequently perturbation of the lipid fraction of the cytoplasmic membrane. This phenomenon can change the fluidity and permeability of membrane resulting in leakage of intracellular materials (Chauhan and Kagn, 2014). Furthermore, the presence of a free hydroxyl group with an adjacent delocalized electron ring enables thymol to exchange proton and thereby inducing conformational modification of the bacteria membrane and eventually cell death (Ben Arfa, et al., 2006).

Cytocompatibility is one of the main criteria of bactericidal component that should be fulfilled. Hence, the MTT assay of thymol-doped dental adhesive was evaluated againstL929 mouse fibroblast cells. According to Figure 5 there are no significant differences ($p > 0.05$) between cell viability values of neat and thymol-

doped dental adhesive with the negative control. For better illustration the morphology of the cells cultured in vicinity of samples was studied through microscopic investigation (Figure 5). Actually, the healthy morphology of cultured cells with spindles shapes and no cell lysis is in good agreement with the finding of MTT assay. In fact, the concentration level of incorporated thymol just exhibited the cytotoxicity effect against Streptococcus mutans bacterial strain and not for fibroblast cells.

5. Conclusions

The thymol is extremely compatible with the methacrylate resin restorative system and completely fulfills all requirements of a good bactericidal component in construction of a ideal enamel bonding system.

Conflict of interest

The authors declare that they have no conflict of interest.

References

10993-5 I. Biological evaluation of medical devices-Tests for in vitro cytotoxicity. Switzerland 2009.

Armstrong S, Geraldeli S, Maia R, Raposo LHA, Soares CJ, Yamagawa J. Adhesion to tooth structure: a critical review of “micro” bond strength test methods. Dent Mater 2010;26:e50-e62.

Balkenhol M, Köhler H, Orbach K, Wöstmann B. Fracture toughness of cross-linked and non-cross-linked temporary crown and fixed partial denture materials. Dent Mater 2009;25:917-928.

Bassolé IHN, Juliani HR. Essential oils in combination and their antimicrobial properties. Molecules 2012;17:3989-4006.

Beigi S, Yeganeh H, Atai M. Evaluation of fracture toughness and mechanical properties of ternary thiol–ene–methacrylate systems as resin matrix for dental restorative composites. Dent Mater 2013;29:777-787.

Ben Arfa A, Combes S, Preziosi-Belloy L, Gontard N, Chalier P. Antimicrobial activity of carvacrol related to its chemical structure. Letters Appl Microbio 2006;43:149-154.

Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, Dorigo EDS. Dental adhesion review: aging and stability of the bonded interface. Dent Mater 2008;24:90-101.

Burujeny SB, Atai M, Yeganeh H. Assessments of antibacterial and physico-mechanical properties for dental materials with chemically anchored quaternary ammonium moieties: Thiol–ene–methacrylate vs. conventional methacrylate system. Dent Mater 2015;31:244-261.

Burujeny SB, Yeganeh H, Atai M, Gholami H, Sorayya M. Bactericidal dental nanocomposites containing 1, 2, 3-triazolium-functionalized POSS additive prepared through thiol-ene click polymerization. Dent Mater 2017;33:119-131.

Chauhan AK, Kang SC. Thymol disrupts the membrane integrity of Salmonella ser. typhimurium in vitro and recovers infected macrophages from oxidative stress in an ex vivo model. Res microbiol 2014;165:559-565.

Cocco AR, da Rosa WLdO, da Silva AF, Lund RG, Piva E. A systematic review about antibacterial monomers used in dental adhesive systems: Current status and further prospects. Dent Mater 2015;31:1345-1362.

Degli Esposti M, Pilati F, Bondi M, de Niederhäusern S, Iseppi R, Toselli M. Preparation, characterization, and antibacterial activity of photocured thymol-doped acrylic resins. J Coat Techno Res 2013;10:371-379.

Designation A. E399-90. Standard test method for plane-strain fracture toughness of metallic materials. American Society for Testing Materials, Philadelphia, Pennsylvania, t990 1997.

Dossot M, Sylla M, Allonas X, Merlin A, Jacques P, Fouassier JP. Role of phenolic derivatives in photopolymerization of an acrylate coating. J Appl Poly Sci 2000;78:2061-2074.

El Zohairy AA, Saber MH, Abdalla AI, Feilzer AJ. Efficacy of microtensile versus microshear bond testing for evaluation of bond strength of dental adhesive systems to enamel. Dent Mater 2010;26:848-854.

Frencken JE, Peters MC, Manton DJ, Leal SC, Gordan VV, Eden E. Minimal intervention dentistry for managing dental caries–a review: report of a FDI task group. Int Dent J 2012;62:223-243.

Fujisawa S, Kadoma Y. Effect of phenolic compounds on the polymerization of methyl methacrylate. Dent Mater 1992;8:324-326.

Kanat B, Cömlekoğlu ME, Cömlekoğlu M, Culha O, Özcan M, Güngör M. Microshear bond strength and finite element analysis of resin composite adhesion to press-on-metal ceramic for repair actions after various conditioning methods. J Adhes Dent 2014;16:63-70.

Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, et al. Antibacterial and antifungal activities of thymol: a brief review of the literature. Food chemistry 2016;210:402-414.

Marshall SJ, Bayne SC, Baier R, Tomsia AP, Marshall GW. A review of adhesion science. Dent Mater 2010;26:e11-e6.

Medina E, Caro N, Abugoch L, Gamboa A, Díaz-Dosque M, Tapia C. Chitosan thymol nanoparticles improve the antimicrobial effect and the water vapour barrier of chitosan-quinoa protein films. J Food Engineer 2019;240:191-198.

Menard KP. Dynamic mechanical analysis: a practical introduction: CRC press; 2008.

Murdoch-Kinch CA, McLEAN ME. Minimally invasive dentistry. J Amer Dent Assoc 2003;134:87-95.

Petersen PE. World Health Organization global policy for improvement of oral health-World Health Assembly 2007. Int Dent J 2008;58:115-121.

Petersen PE, Ogawa H. Prevention of dental caries through the use of fluoride–the WHO approach. Community Dent Health 2016;33:66-68.

Placido E, Meira JB, Lima RG, Muench A, de Souza RM, Ballester RY. Shear versus micro-shear bond strength test: a finite element stress analysis. Dent Mater 2007;23:1086-1092.

Seow YX, Yeo CR, Chung HL, Yuk H-G. Plant essential oils as active antimicrobial agents. Critical reviews in food science and nutrition 2014;54:625-644.

Standard I. ISO 4049 Polymer based filling, restorative and luting materials. International Organization for Standardization 2000:1-27.

Soderholm KJ. Review of the fracture toughness approach. Dent Mater 2010;26:e63-e77.

Van Ende A, De Munck J, Mine A, Lambrechts P, Van Meerbeek B. Does a low-shrinking composite induce less stress at the adhesive interface? Dent Mater 2010;26:215-222.

Watanabe H, Khera SC, Vargas MA, Qian F. Fracture toughness comparison of six resin composites. Dent Mater 2008;24:418-425.

Captions to tables and figures:

Figures:

Figs. 1a-b Flexural properties of adhesives specimens, **a)** Fracture toughness of samples, **b)** According to the analysis of variances, the difference between quantities with similar superscripts (A,B) is not significant ($p>0.05$).

Figs. 2a-b Loss tangent vs. temperature of the specimens, **a)** the images of photocured discs of dental adhesives.

Figs. 3a-c Microshear bond strength of the adhesives. Porcelain Bonding (Bisco Inc, USA) is a commercially available enamel bonding agent, **a)** According to the analysis of variances, the difference between quantities with similar superscripts (a) Is not significant ($P>0.05$) The typical SEM micrographs of the enamel surface before and, **b)** after microshear bond fracture, **c)** The fracture are in microshear bond strength test showing an adhesive failure mode adhesive-enamel interface.

Tables:

Table 1. Different formulations of enamel bonding systems and their viscoelastic properties, Log reduction in CFU of bacteria and degree of conversion of methacrylate functional group.[1,2] [1)]For data of DC%, standard deviation value is shown in parenthesis. [2)]According to analysis of variances ($P\leq0.05$) the difference between quantities with similar superscripts (a) is not significant.

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Table 1. Different formulations of enamel bonding systems and their viscoelastic properties, Log reduction in CFU of bacteria and degree of conversion of methacrylate functional group.[1,2] [1)]For data of DC%, standard deviation value is shown in parenthesis. [2)]According to analysis of variances (P≤0.05) the difference between quantities with similar superscripts (a) is not significant.

z	BisGMA/ TEGDMA	Thymol	Log reduction in CFU of Mutans (1 day aged)	Log reduction in CFU of Mutans (4 months aged)	T_g (°C)	$T_{g1/2width}$ (°C)	DC (%)
Neat adhesive	100 wt%	-	-	-	98.81	62.26	60.22(2.51)$_a$
thymol-doped adhesive	95 wt%	5 wt%	86%	78%	111.38	98.84	63.09(3.75)$_a$

Figures:

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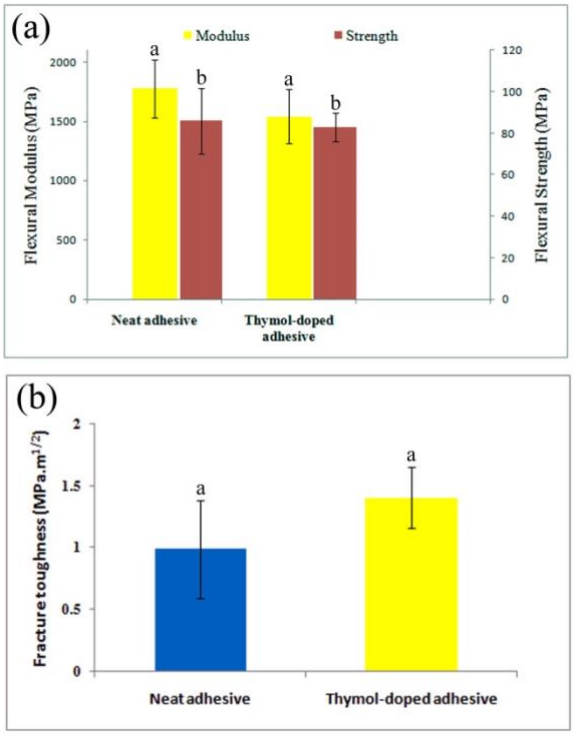
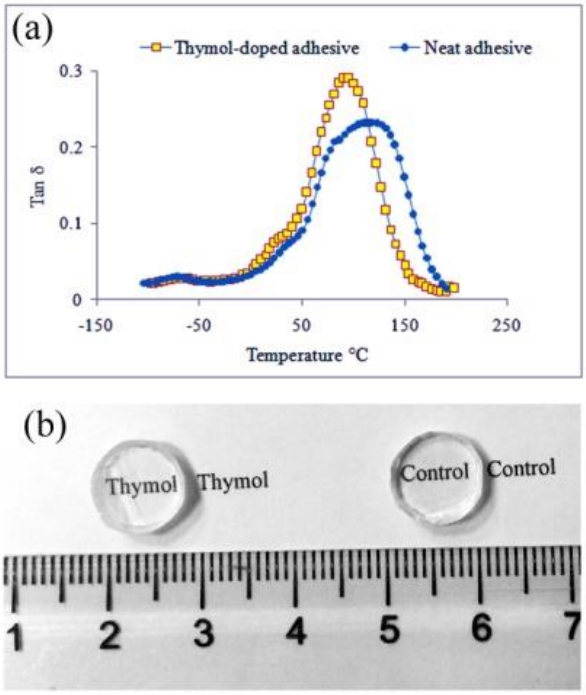


Figs. 2a-b Loss tangent vs. temperature of the specimens, **a)** the images of photocured discs of dental adhesives.



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